LIVE ATTENUATED VACCINES

Field of the Invention

The present invention relates to attenuated strains of pathogenic bacteria, particularly of *Neisseria meningitidis*. These strains are particularly useful as live attenuated vaccines.

Background to the Invention

Bacterial infections are a significant cause of disease throughout the world. Many bacterial infections can prove fatal, particularly in vulnerable individuals such as infants, children, the elderly or those who are immunocompromised by other diseases or through injury.

Bacterial diseases which are still prevalent include meningococcal meningitis, caused by *Neisseria meningitidis* and gonorrhoea, caused by the related organism *Neisseria gonorrhoeae*. Both these organisms are gram-negative bacteria. Other gram-negative bacteria include *Helicobacter pylori* (associated with stomach ulcers), *Salmonella spp.*, e.g. *Salmonella typhi* or *Salmonella typhimurium*, enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), verotoxigenic *E. coli* (VTEC), *Vibrio cholerae*, *Shigella spp.*, (all of which cause gastric infections), *Haemophilus influenzae* and *Bordetella pertussis*, which may cause meningitis and whooping cough respectively, particularly in children, and *Pseudomonas aeruginosa*, which can cause infection in infants, children and adults and is particularly a problem in individuals who have cystic fibrosis.

The goal of any vaccine development programme is to provide a vaccine which is safe, capable of providing cross-group protection and capable of providing long-term protection.

Traditionally there are three main groups of vaccine types. (1) Killed vaccines, where the killed pathogen is used directly, (2) Defined vaccines where purified components of the pathogen are used and (3) Live attenuated vaccines, where the live pathogen is used which has been rendered unable to grow for extended periods in the host. Each of these vaccine types presents different advantages and disadvantages.

Killed vaccines are superficially attractive, particularly from the safety point of view. However such vaccines can not proliferate or colonise, nor is there any dynamic gene expression, which might result in the production of useful antigens which elicit an immunoprotective response. Indeed the production of the killed vaccine usually results in the

exposure of internal antigens such as lipid A which may compromise its use due to induction of adverse reactions in the host. In most cases the cause(s) of these deleterious effects are unknown.

In view of such problems, many researchers have endorsed a defined or subunit vaccine approach where those antigens which appear to elicit the "best immune response" in the host are purified and used directly as a vaccine. This approach again avoids the use of live bacteria which is also attractive from the safety and regulatory point of view. The chosen components may be subunits of actual virulence proteins or somatic proteins. There are obvious advantages to this approach:- (1) The vaccine is totally defined and the immunogenicity of each purified component protein is understood. (2) Any toxic component which may elicit adverse effects can be eliminated from the vaccine. (3) If the same strain and procedures are used there is the potential for vaccines made in different locations being consistent. (4) Since the vaccine is composed of purified cell components, it is likely to be relatively stable under normal conditions of storage.

There are also major disadvantages to this approach. (1) Only selected cell components are used and these may not on their own be sufficient to elicit good protective long term immunity. (2) The most important antigens for full protection may be unidentified and/or only be expressed under *in vivo* conditions and therefore will be absent from the vaccine. (3) Vaccine production entails expensive protein and/or cell component purifications reliant on advanced technology. (4) Protein antigens most important for the host immune response will most probably be secreted toxins or surface exposed outer membrane proteins which may undergo some degree of denaturation during purification. Denatured proteins will lose conformational epitopes that may be important and if used in vaccines may elicit immune responses to epitopes which are not present in the native, membrane associated protein. (5) Purified outer membrane proteins may require an additional, expensive renaturation step, and possibly re-insertion into a membrane vesicle carrier to make them useful as a vaccine. (6) Renaturation of proteins *in vitro* is difficult and never completely successful. Aberrant epitopes may remain and the important epitopes of the protein not be reformed even when inserted into membrane vesicles. Therefore it is probably naive to expect that a few proteins purified apart from their normal membrane environment would elicit a good cell mediated response in the host. (7) Association of individual outer membrane proteins in the membrane surface of the intact organism appears important for the induction of an immune response to the

individual proteins. Proteins removed from such associations may not elicit protective/bactericidal responses even though they may do so in the intact organism. This may be due to conformational epitopes being formed between two or more associated proteins. (8) The normal association of proteins within the membrane may generate important antigenic epitopes which could be lost when individual components are purified. Thus in many respects, defined/subunit vaccines are far from ideal.

Live attenuated vaccines offer the best prospect for a vaccine which will give similar protection to that seen with the natural disease. The advantages of a live attenuated vaccine are numerous. (1) The whole live organism is used, rather than dead cells or selected cell components which may exhibit modified or denatured antigens. Therefore the potential to elicit a good protective long term immunity should be higher. (2) The most important antigens for full protection need not be identified, since the vaccine will only have specific modifications for attenuation it will still be able to express these important antigens under *in vivo* conditions. (3) Production costs should be a fraction of more intensive and expensive vaccine production procedures, since there is no protein/subunit purification, protein refolding, or down stream processing required except the minimum essential for the vaccine application. (4) Protein antigens in the outer membrane will maintain their tertiary and quaternary structures. (5) The associations between individual proteins in the membrane surface of the vaccine strain which may be important for the generation of conformational epitopes between two or more associated proteins will be maintained. (6) The fluid structures of these proteins will also be maintained in the attenuated vaccine strain. (7) Since the vaccine is live it will display dynamic gene expression in response to environmental changes and express those proteins which are only present or enhanced *in vivo* in the wild type pathogen. (8) The host responses to the attenuated strain would mimic that expected for the pathogen since the attenuation process would not affect the expression of the important immunogenic proteins. (9) Production of live attenuated vaccines is much simpler. As long as recommended growth conditions are adhered to and a standard stock of the vaccine strain is maintained and used as the initial inoculum vaccines should be uniform in composition and efficacy. (10) With improved methods of freeze drying and storage the vaccine should be stable and viable at normal ambient temperatures. (11) Live attenuated vaccines can in addition, be important vehicles for the delivery of other antigens/immunogens for example toxins. This would be particularly important where the immunogen which confers complete protection is a secreted toxin, e.g. diphtheria toxin,

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pertussis toxins or cholera toxin B subunit.

However, one difficulty with the production of live attenuated vaccines is that during bulk culture the organism does not always express some proteins which are important for host protection. In addition, once such vaccines have been administered to the host, the vaccine strain does not have the optimum time or metabolic resources during its limited colonisation to express these protein *in vivo* to a sufficient level to confer protection.

Summary of the Invention.

The present invention is based on the finding that altering the regulation of the ferric uptake regulation (*fur*) gene in *N. meningitidis*, such that its expression is independent of the iron concentration in the environment of the bacterium, enhances the expression of important protective antigens when the bacterium is grown in culture. This has important implications for the manufacture of live vaccines since the increased expression of these protective antigens during the manufacture process will increase the efficacy of the live vaccine when administered to an animal or human subject.

Thus in a first aspect the invention provides an attenuated bacterium in which the native *fur* gene, or homologue thereof, is modified such that the expression of the *fur* gene product, or homologue thereof, is regulated independently of the iron concentration in the environment of the bacterium. The attenuated bacterium is preferably a gram-negative bacterium, for example selected from the genera *Neisseria, Helicobacter, Salmonella, Escherichia, Vibrio, Shigella, Haemophilus, Bordetella, Pseudomonas, Yersinia* and *Brucella.* More preferably the bacterium is selected from the group consisting of *N. meningitidis, N. gonorrhoeae, Helicobacter pylori, Salmonella spp.* (including *S. typhi* and *S. typhimurium*), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC) enterohaemorrhagic *E. coli* (EHEC), verotoxigenic *E. coli* (VTEC), *Vibrio cholerae, Shigella spp., Haemophilus influenzae, Bordetella pertussis* and *Pseudomonas aeruginosa.* Especially preferred are *N. meningitidis* and *N. gonorrhoeae*, most preferably *N. meningitidis.*

Preferably, the bacterium has been attenuated by mutation of a gene essential for the production of a metabolite or catabolite not produced by a human or animal. The mutated gene is most desirably an *aro* gene such as an *aroA, aroB, aroC, aroD* or *aroL* gene or a gene of the *pur* or *pyr* pathways such as *purA, purB, purE, pyrA, pyrB* or *pyrE.* The *aroB* or *aroL* gene is preferred, in particluar for *N.meningitidis.* When the bacterium is *N. meningitidis* or *N.*

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gonorrhoeae, it may also carry a *gal*E mutation. Optionally the *gal*E mutation is accompanied by modification or elimination of expression from the *opc* gene. Attenuation mutations may be provided in one or more, for example two or three, genes.

In yet another preferred aspect, the bacterium further comprises a *rec*A mutation. Optionally, in addition to or instead of the *rec*A mutation, the bacterium also comprises a mutation in a gene responsible for the uptake of DNA. These gene mutations will provide attenuated strains in which the possibility of homologous recombination with DNA from wild-type strains has been minimised. Such a gene may include the *com*A pilin gene of *N. gonorrhoeae* (Molecular Microbiology (1993) **10**: 699-712) or its homologues in other species - particularly *N. meningitidis* - which may be obtained by methods analogous to those described herein for other genes.

Further modifications may be made for additional safety. For example the bacterium may additionally comprise a mutation in which expression of toxin genes has been modified or eliminated. In particular, for *Neisseria* species, the RTX (repeats-in-toxin) toxin gene may be modified or eliminated. This is accomplished by deleting the *frp*A gene in the *frp* polycistronic locus, while maintaining the correct reading frame for the polycistron to retain expression of outer membrane proteins which may be important for the efficacy of the vaccine.

Certain bacteria such as *N. meningitidis* and others mentioned above are known to produce membrane vesicles. This is caused by aberrant cell division resulting in DNA-less cells (or mini-cells) which bud from the dividing bacteria. Such membrane vesicles, particularly of *N. meningitidis*, form a further aspect of the invention. Production of such vesicles is enhanced by mutation in the locus homologous to the *E. coli min*B locus and thus in a further aspect of the invention mutations in these genes may be made.

Particularly preferred strains of *N. meningitidis* have the genotypes: Δ*aroB, lac:fur* fusion; *aroB, lac:fur* fusion, *rec*A; *aro*B, *gal*E, *lac:fur* fusion, *rec*A; *aro*L, *lac:fur* fusion, *recA*; *aro*L, *gal*E, *lac:fur* fusion, *rec*A. The strains may further comprise at least one of a *min*B mutation, an RTX negative phenotype, modified expression of the *opc* gene and/or an additional attenuation mutation in a second *aro* gene, *pur*B, *pur*E, *pyr*A or *pyr*B.

The invention further provides a vaccine preparation which comprises a bacterium or a vesicle preparation of the invention together with a pharmaceutically acceptable diluent or carrier. Such vaccines may be used in a method of treatment of the human or animal body. Such a method may comprise protecting an individual against a bacterial infection by

administering to an individual an effective amount of a vaccine composition comprising an attenuated bacterium, according to the present invention, normally associated with said infection.

The invention further provides a process for preparing a vaccine composition comprising an attenuated bacterium according to the invention which process comprises (a) inoculating a culture vessel containing a nutrient medium suitable for growth of said bacterium; (b) culturing said bacterium; (c) recovering bacteria from the culture and (d) mixing said bacteria with a pharmaceutically acceptable diluent or carrier.

The present invention also provides a method for producing a bacterium according to the invention which method comprises modifying the native *fur* gene, or homologue thereof, of an attenuated bacterium such that expression of said *fur* gene or homologue is regulated independently of the iron concentration in the environment of the bacterium. Preferably, said bacterium has been attenuated by mutation of at least one gene essential for the production of a metabolite or catabolite not produced by a human or animal, more preferably by a mutation of an *aro* gene such as an *aro*B gene and/or *aro*L gene and/or a gene of the *pur* or *pyr* pathways, in particular a *purE*, *purB*, *pyrA* and/or *pyrB* gene.

Brief Description of the Drawings.

Figure 1 illustrates the construction of the suicide/transformation vector pGIT5.
Figure 2 shows the primers used for the amplification of the *aroB* gene from *Neisseria meningitidis* and construction of an *aroB* deletion mutant.
Figure 3 illustrates the construction of an *aroB* deletion mutant
Figure 4 illustrates the construction of an *asd* mutant
Figure 5 illustrates the production of a *fur/lac* fusion strain.

Detailed Description of the Invention.

The term "vaccine" as used herein means an agent used to stimulate the immune system of a vertebrate, particularly a warm-blooded vertebrate including humans, so as to provide protection against future harm by an organism against which the vaccine is directed. The immune system can provide two types of responses to foreign organisms - cell-mediated (T cell) and humoral (B cell/antibody) responses. Both types of responses can be stimulated by a

suitable vaccine to produce a primary immune response which leads to the development of immunological memory, providing protection against a subsequent infection by a pathogen. Stimulation of the cell-mediated immune system results in the development of naive T cells into effector T cells which are able to react much more quickly to future challenges. Stimulation of B cells of the humoral immune system leads to the production of antibodies, desirably neutralising antibodies/ bacteriocidal antibodies, directed to epitopes found on or in the attenuated bacterial strain. The antibody so produced may be any of the immunological classes, such as the immunoglobulins A, D, E, G or M. Vaccines which stimulate the production of IgA are of particular interest since this is the principle immunoglobulin produced by the secretory system of warm-blooded animals, and the production of mucosal antibodies will help prevent infection or colonisation of the nasopharynx, which is one natural route of infection of bacteria including *N. meningitidis*. However, this and other organisms may also be infectious via the bloodstream and thus an IgM and IgG response will also be desirable.

The bacterium of the invention may be in isolated form. This is usually desirable when the bacterium is to be used for the purposes of vaccination. The term "isolated" means that the bacterium is in a form in which it can be cultured, processed or otherwise used in a form in which it can be readily identified and in which it is substantially uncontaminated by other bacterial strains, for example non-attenuated parent strains or unrelated bacterial strains. The term "isolated bacterium" thus encompasses cultures of a bacterial mutant of the invention, for example in the form of colonies on a solid medium or in the form of a liquid culture, as well as frozen or dried preparations of the strains.

The term "mutation" and the like refers to a genetic lesion in a gene which alters the levels of expression of the gene or the activity of the gene product. Such a mutation may exert its effect on the levels of expression of a gene product at any stage from the level of transcription to the level of translation and protein stability. Alternatively, a mutation may cause in a change in the coding sequence such that the resulting gene product has altered activity, generally reduced activity. For example, an "inactivating mutation" would render the gene non-functional. The term "inactivating mutation" thus envisages deletion of the entire gene or substantial portions thereof, and also point mutations in the coding sequence which result in truncated gene products unable to carry out the normal function of the gene. Where deletion of a portion of a gene is carried out, it may be desirable to introduce frame shift mutations downstream of the deleted region to remove the possibility of small sections of the

gene being expressed.

A mutation introduced into a bacterium of the invention will generally be a non-reverting attenuating mutation. Non-reverting means that for practical purposes the probability of the mutated gene being restored to its normal function is small, for example less than 1 in 10^6 such as less than 1 in 10^9 or even less than 1 in 10^{12}. However despite this, it may be considered desirable that a bacterium for use as a live attenuated vaccine should carry at least two attenuating mutations. Usually these mutations will be in separate genes.

An "attenuating mutation" is a mutation in a gene which is required for the growth of a bacterium. Such a gene is thus usually a metabolic, for example a catabolic or biosynthetic, gene. Preferred attenuating mutations are made in genes which are required for the production of an essential nutrient or intermediate which is not available in the tissues of the human or animal host in which the bacterium is normally pathogenic. Thus the attenuated bacterium will not be able to simply obtain the essential nutrient or intermediate from the infected host.

A bacterium of the invention will be an attenuated form of a pathogenic bacterium. The term "pathogenic" means that the bacterium is capable of causing disease in a human or animal (especially avian or mammalian, including human) host, although in some cases the bacterium may be carried asymptomatically by healthy individuals. Examples of the latter type include *N. meningitidis* and *S. aureus*.

Production of Bacteria

The survival of any bacterium in a given environment depends on its ability to obtain all its growth requirements in competition with other resident organisms. It must express those proteins which are essential for growth and replication if it is to be competitive and survive. Strains which carry a particular mutation or combination of mutations may be made from a species of bacteria in accordance with the present invention. These strains will carry particular genetic lesions or mutations which render the organism auxotrophic for an essential nutritional requirement. If the organism is unable to acquire this nutrient from its environment it will not proliferate and will die once endogenous levels are exhausted. If such mutations are incorporated into normally pathogenic bacteria and the essential nutrient or its intermediates are absent from the tissues of the host then that mutant organism will be attenuated and unable to establish a normal infection, if colonisation is a component of the infection process.

Bacterial strains according to the invention may be constructed using recombinant DNA

methodology which is known *per se*. In general, bacterial genes may be mutated by a process of targeted homologous recombination in which a DNA construct containing a mutated form of the gene is introduced into a host bacterium which it is desired to attenuate. The construct will recombine with the wild-type gene carried by the host and thus the mutated gene may be incorporated into the host genome to provide a bacterium of the present invention which may then be isolated.

Various assays are available to detect successful recombination. In the case of attenuations which mutate a target gene necessary for the production of an essential metabolite or catabolite compound, selection may be carried out by (i) screening for bacteria unable to grow in the absence of such a compound; (ii) genetic methods to determine the deletion of the gene and/or (iii) showing that a genetically determined mutant can not grow in human fluids, for example blood or cerebrospinal fluid.

The mutated gene may be obtained by cloning the wild-type gene from the bacteria which is to be attenuated, or from a closely related species, and manipulating it by standard recombinant DNA techniques. These include introducing deletions into the gene, e.g. by digesting with a restriction enzyme which cuts the coding sequence twice to excise a portion of the gene and then religating under conditions in which the excised portion is not reintroduced into the cut gene. Alternatively frame shift mutations, which alter the reading frame of the DNA sequence, may be introduced by cutting with a restriction enzyme which leaves overhanging 5' and 3' termini, filling in and/or trimming back the overhangs, and religating. Similar mutations may be made by site directed mutagenesis. These are only examples of the types of techniques which will readily be at the disposal of those of skill in the art. Another example is PCR of flanking regions adjacent to the target gene followed by ligation to form a deletion mutation construct.

The cloning of the wild-type gene may be undertaken using PCR cloning methods which are also known *per se* in the art. Such methods require knowledge of at least part of the sequence of the gene which is to be mutated. The sequence may be obtained by reference to databases such as Genbank. Where a gene sequence is not known for a particular organism, consensus sequences may be determined by aligning sequences of corresponding genes of other, related, organisms and determining regions which are most conserved. For example, the *aro*, *pur* and *pyr* genes of *N.meningitidis* can be isolated by screening the *N.gonorrhoea* raw database for sequences that have similarity to *E.coli* proteins. The appropriate gene sequences

can be identified and primers designed for amplification of the corresponding genes of *N.meningitidis*. In general, many genes, especially those of metabolic and catabolic pathways are well conserved between gram-negative bacteria and appropriate PCR primers can be determined without difficulty.

The above techniques are exemplified further in the Examples below and can be readily adapted to provide other embodiments of the invention. Reference may also be made to, for example, Sambrook *et al.*, "Molecular Cloning - A Laboratory Manual" (Cold Spring Harbor, 1989) or "Short Protocols in Molecular Biology - A Compendium of Methods from Current Protocols in Molecular Biology" eds. Ausubel *et al.* (John Wiley & Sons, 1992).

Attenuating Mutations.

An attenuating mutation can, for example, be made in a gene encoding a component of a metabolic pathway so that the growth of the attenuated bacterium is inhibited due to a lack of an essential metabolic intermediate of a particular metabolic pathway. Growth of such attenuated bacteria can then be achieved in culture by supplying the essential metabolic intermediate in the culture medium. However, when such attenuated bacteria are administered to a human or animal host for vaccination purposes, it is clearly desirable that the host itself is not able to supply the essential metabolic intermediate required by the attenuated bacterium for optimum growth. Consequently, attenuating mutations are preferably made in genes essential for the production of a metabolite, for example an anabolite or catabolite, not available in the human or animal host which is to be vaccinated.

Particular examples of pathways whose genetic components may be inactivated by mutation include 1. aromatic amino acid biosynthetic pathways, 2. purine biosynthetic pathways and 3. pyrimidine biosynthetic pathways.

1. Aromatic ring biosynthetic pathways – The shikimic acid pathway

This pathway is involved in the biosynthesis of virtually all aromatic compounds but is not present in mammals. It is therefore particularly preferred that mutations are made in genes encoding components of this pathway. The enzymes catalysing the various reactions of the shikimic acid pathway (and their corresponding genes) include 3-dehydroquinate synthase (*aro*B), 3-dehydroquinate dehydratase (*aro*D), shikimate dehydrogenase (*aro*E), shikimic acid kinase I (*aro*K), shikimic acid kinase II (*aro*L), 5-enoylpyruvyl shikimic acid 3-phosphate

(*aro*A) and chorismate synthase (*aro*C).

A lesion in any of the genes of the shikimic acid pathway would render the bacterium auxotrophic for the essential nutrient chorismate. If the lesion occurs in a gene of the pathway prior to shikimic acid kinase I (*aro*K) but not in any of the downstream genes the bacterium will also be auxotrophic for the essential nutrient shikimate. Since these compounds are essential for aromatic amino acid biosynthesis in gram-negative and some gram-positive organisms and are absent from mammalian or other vertebrate tissues, mutants would undergo lysis after about three rounds of division in such tissues. Such a mutation will limit the proliferation of the bacteria in host tissues but will not affect their growth during vaccine production since the essential nutrients or aromatic amino acids can be provided exogenously during bulk culture of the vaccine strain.

The invention provides, in a particularly preferred embodiment, a live attenuated vaccine which is effectively attenuated by construction of specific deletion and frame-shift mutations initially in the *aro*B gene. The *Neisseria gonorrhoeae aro*B gene has been cloned and its nucleotide sequence deposited in Genbank as Accession No. AJ002783.

It is also particularly preferred that an attenuated bacterium of the invention has an inactivating mutation in the *aro*L gene, in addition to or instead of an inactivating mutation in the *aro*B gene. The *E. coli aro*L gene is described in Biochem J. (1986) **237(2)**: 427-437 (Genbank Accession No. X04064).

Functional inactivation of the *aro*A gene is preferably a secondary mutation once a primary mutation in one of the other *aro* genes (or one of the other genes described below) has been carried out. The *E. coli aro*A gene is described in FEBS Lett. (1984) **170**: 59-63 (Genbank Accession No. X00557).

Mutations in *aro*C and *aro*D have been described in EP-A-322 237. The *E.coli aro*C gene is described in Biochem J. (1988) **251(2)**: 313-322 (Genbank Accession No. Y00720). The *Neisseria gonorrhoeae aro*D gene is described in Mol. Gen. Genet. (1997) **254(5)**: 479-485 (Genbank Accession No. U39803). The *Neisseria meningitidis aro*E gene is described in Mol. Microbiol. **23(4)**: 799-812 (1997) (Genbank Accession No. U82841).

2. Purine biosynthetic pathways

Mutations in any of the genes in the *pur* pathway (e.g. *pur*B (encoding adenylosuccinate lyase) or *pur*E (encoding 5'-phosphoribosyl-5-amino-4-imidazole carboxylase)) will render the

organism auxotrophic for purines, essential constituents of nucleic acids. Many of the intermediates of the pathway are unavailable in mammalian or other vertebrate tissues.

3. Pyrimidine biosynthetic pathways

Mutations in any of the genes in the *pyr* pathway (e.g. *pyr*A (encoding carbamyl phosphate synthetase) or *pyr*B (encoding aspartate transcarbamylase)) will render the organism auxotrophic for pyrimidines, essential constituents of nucleic acids. Many of the intermediates of the pathway are unavailable in mammalian or other vertebrate tissues.

Other genes which may be mutated include the *asd* gene which encodes aspartate-semilaldehyde dehydrogenase. Mutations in *asd* would not be made as a primary mutation and would instead be made subsequent to a primary mutation in one of the other genes described above. The *N. meninigitidis asd* gene is described in Gene (1993) **129(1)**: 123-128 (Genbank Accession No. Z14063).

Inactivating mutations may be made in only one of the genes described above (or another suitable gene) or alternatively, mutations in multiple genes may be employed, for example with mutations in the *aro*B gene as the primary mutation followed by mutations in at least one of the other genes. A double or triple mutant may be useful from the safety point of view since it will be more attenuated, but such a double mutant may also be less efficient as a vaccine. Strains with different levels of attenuation may be assessed during trials for their degree of efficacy and any side effects. Mutations of any of these genes may be accomplished, for example, in a similar way to that described for *aro*B in the Examples.

Mutation to maximise exposure of important epitopes

The *galE* gene codes for UDP-galactose-4-epimerase. In *Neisseria* this enzyme does not appear to have a role as part of the galactose biosynthesis pathway, but it is involved in the incorporation of galactose into the phase-variable oligosaccharide side chains of LPS/LOS. *N. meningitidis* contains two chromosomal *galE* sequences, *galE1* and *galE2* at separate loci. *GalE1* is complete, expressing a functional gene product, while *galE2* is truncated, and lacks homology over the first third of the protein. The *galE* gene and its role in biosynthesis of LOS/LPS is described in Molecular Microbiology (1993) **10**: 361:369.

A mutation in the *galE* gene denudes and shortens the LPS of galactose residues. Thus,

a *galE* deletion exposes the constant region of the LPS to the immune system, which leads to the generation of a cross-protective response. The reduction in the length of LPS may reduce the capsule on the surface of the organism. A further effect of a *gal*E mutation and the resulting short LPS is that outer membrane proteins exposed on the cell surface will become more accessible to the hosts immune system potentially leading to an improved immune response to the vaccine strain. Thus incorporation of a *gal*E mutation, rendering the gene non-functional, into a *Neisseria* strain of the invention is preferred.

In addition, without the incorporation of galactose into the LPS the potential autoantibody-producing epitopes of LPS will not be incorporated. These epitopes are normally found on host epithelial cells and in many host secretions. Thus their removal from vaccine strains will greatly reduce the danger of immunopathological effects of the vaccine.

It has also been shown in bacteria, particularly *N. meningitidis*, that the *galE* mutation results in an increase in *opc*-mediated invasion. For a vaccine intended for nasal delivery, this may be advantageous in stimulating the initial host tissue response and subsequent mucosal immunity, while other mutations (such as in the *aro*B gene) would limit the proliferation of invading bacteria to approximately three rounds of division, precluding the possibility of a systemic infection. Also there is some evidence that *gal*E mutants are serum sensitive and therefore would be rapidly killed if they entered the hosts circulatory system.

A mutation in a capsule gene, *cap*, may also result in increased exposure of epitopes. That may in turn result in an increased immune response to the attenuated baterium.

Invasion/adhesion.

A *gal*E mutation will expose the Opc protein thus making the vaccine strain able to colonise and invade into the blood stream more efficiently. This would be important to get a good mucosal and serum immune response in the limited time that the strains survive in the human body. There are two possible modifications to *opc* that may be made in accordance with one embodiment of the invention.

If there is excessive adhesion or invasion with the vaccine strain, then *opc* expression may be modified or even eliminated entirely. Where *opc* expression is found to be important for vaccine efficacy but is variable in expression, then *opc* may be placed under the control of another promoter e.g. *cat*, which can be more readily controlled. The *opc* opacity protein may be found by reference to the EMBL/GenBank/DDBJ databases. 1993. Accession number

Z14063 (Microb. Pathog (1991) **11**:249-257).

Mimicking *in vivo* expression of iron regulated proteins

One failing of live attenuated vaccines in the past has been that during bulk culture the organism has not expressed some proteins which are important for host protection. Such proteins include iron regulated proteins, the production of which is regulated by the *fur* (ferric uptake regulation) gene. In addition, once such vaccines have been administered to the host, the vaccine strain has not had time or metabolic resources during its limited colonisation to express these proteins *in vivo*.

To overcome these problems, the present invention provides vaccine strains which have been modified such that many of the proteins which are likely to be important immunogens, e.g. the iron regulated proteins, are already expressed on the cell surface during bulk culture. This is achieved by altering the regulation of the ferric uptake regulation *(fur)* gene (EMBL/GenBank/DDBJ databases. 1993. Accession number L19777, Mol. Microbiol (1994) **11(4)**: 725-737 (*Neisseria*); Mol. Gen. Genetics (1985) **200**: 110-113 (*E. coli*)).

The *fur* gene may be modified to produce its gene product independently of the iron concentration in the environment of the bacteria by any suitable means. In this context, the term "gene" is intended to encompass both the coding sequence of the fur protein together with any transcriptional/translation control sequences present, including promoters, transcriptional stop sequences, ribosome binding regions and other translation regulation elements. Thus, modifications may be made to the coding sequence and/or the transcriptional/translation control sequences. For example, the promoter sequences of the gene may be determined (e.g. by reference to the database sequence and/or sequencing this region using PCR primers directed upstream from the coding region) and the promoter regions responsible for iron concentration-dependent regulation may be modified by mutation, including deletion, insertion and/or substitution. One such identified promoter region is the fur box. Since fur expression may be required for positive regulation of some essential genes in *Neisseriacae* and complete inactivation of the *fur* gene is often lethal to the bacterium, the minimum levels of expression from the modified *fur* gene will preferably be sufficient to allow viable growth of the bacterium. In particular, levels of expression from the modified fur gene are preferably at least equal to those obtained using the native *fur* gene in the presence of low levels of ferric ions, i.e. the maximal repressed state.

Alternatively the endogenous *fur* gene may be placed under the control of another promoter entirely. Such a heterologous promoter may be any promoter which is regulated independently of iron concentration and which functions in the host bacterium. Preferably said promoter comprises a bacterial promoter such as a promoter derived from that bacterium or a member of the same family, or from a bacteriophage. Suitable promoters include both constitutive promoters such as that of the chloramphenicol resistance gene and regulatable promoters such as the promoters of catabolic, metabolic and biosynthetic genes, including those involved in the regulation of amino acid biosynthesis (which are induced when a required amino acid is absent or at a low concentration) or those involved in the metabolism of sugars (which are induced by the presence of the appropriate sugar). The *trp* promoter and the *lacZ* promoter are particular examples of the above, and the latter is especially preferred.

The promoter region of the endogenous fur gene is conveniently modified by homologous recombination with a suitable construct. The construct may comprise all or part of the *fur* gene together with the desired replacement promoter sequences provided that recombination results in modification of the endogenous *fur* gene. Alternatively, the endogenous *fur* gene may be inactivated, and a complete *fur* gene together with the desired replacement promoter sequences introduced into the bacterial genome or maintained as an extrachromosomal plasmid. This will ensure that even in the presence of iron little *fur* gene expression occurs, although the heterologous promoter will allow sufficient expression of any essential genes positively regulated by fur.

In a particularly preferred embodiment of the invention, to overcome the problems of regulating *fur* and also to mimic *in vivo* levels of expression of iron regulated genes, *fur* gene expression is placed under the control of the *lacZ* promoter/*lacI* repressor which confers positive regulation of -galactosidase in the presence of lactose. Preferably, the I^q mutation in *lacI* is included which increases the basal level of expression ten-fold. This provides for sufficient production of fur to allow expression of essential genes and also allows the control of fur expression to be regulated in response to intracellular lactose concentrations.

Mutations may also be made in the coding sequence of the *fur* gene, which encodes the fur protein. By way of example, a mutation can be made which modulates the affinity of the fur protein for ferric ions. Preferably, such a mutation would abolish or substantially reduce the affinity of the fur protein for ferric ions. In this way, the fur proteins transcriptional regulatory functions would be independent of the iron concentration in the environment of the bacterium.

Preferably, an attenuated *asd* mutant is used and its *fur* gene replaced with a *fur* gene under the control of the *lacZ* promoter/*lacI* repressor. The resulting low levels of the fur protein would enable the negatively regulated fur-controlled genes to be switched on, mimicking iron restricted conditions. The strain would therefore be fully attenuated but would fully express all iron-regulated proteins before being administered to the recipient without the need for iron-restricted growth conditions. The host would then be able to mount an immune response that would mimic that in response to the wild-type pathogen.

Thus placing *fur* under the regulation of the *lacZ* promoter is preferred. This allows its expression only in the presence of lactose or one of the non-metabolisable lactose analogues such as IPTG. Since the *lac* inducer dissociates from the *lac* operator in the presence of lactose, fur expression can be reduced down to basal levels by growing the strain in the absence of lactose. This effectively up-regulates all fur regulated genes mimicking growth in low iron, *in vivo* conditions. The result will be that in the absence of lactose, the bacteria will respond as if they are experiencing iron restricted conditions, producing the iron regulated proteins normally expressed *in vivo*, without the use of large quantities of iron chelators. It is thus possible to control *fur* expression independent of the iron concentration in the medium. *Fur* expression will only be at "trickle through" basal levels in the absence of lactose. The maintenance of this basal level of expression is thought to be important for cell viability.

Therefore, in this embodiment of the invention, when the vaccine is administered to the host, many of the important proteins will already be present on the bacterial surface. This will relieve the metabolic burden that the bacterium will experience *in vivo*, freeing the resources of the bacterium to express other *in vivo* regulated proteins which are not iron regulated but are important for host protection.

The production of attenuated strains of bacteria in which the expression of *fur* has been modified as described above is a generally applicable technique. Thus any normally pathogenic bacterium which has a genome comprising a *fur* gene or homologue thereof, which bacteria is attenuated by deletion or modification of a gene essential for growth in a host in which the bacteria is pathogenic, may be modified as described above so that it produces the *fur* gene product, or homologue thereof, independently of the iron concentration in the environment of the bacteria. *Fur* homologues can be identified by sequence comparison with other known *fur* gene sequences from, for example *E. coli* and *N. meningitidis*. Preferably, such homologues are substantially homologous to another known *fur* gene. In particular it is preferred that

homologues have sequence identity of at least 60% at the amino acid level, more preferably 70%, over a stretch of at least 100 amino acids. *Fur* homologues can also be identified on the basis of function. The product of the *fur* gene in *Neisseria* controls the expression of numerous genes including those encoding toxins and iron uptake components, and is responsive to ferric ion concentrations. Thus genes identified in other bacteria which encode transcription factors which also have these ferric iron-responsive properties may be used.

Mutations to achieve genetic isolation and stability.

Another consideration of live attenuated vaccines which has to be addressed is the genetic stability of the strain. The strain must not be able to revert to a wild-type phenotype, nor must it lose the ability to express genes important for host protection. Genetic stability can be increased by introducing a deletion in the *recA* gene. This gene enables the organism to repair its DNA by homologous recombination. The *rec*A mutation knocks out homologous recombination - the process which is exploited for the construction of the mutations. Once the *rec*A mutation has been incorporated the strain will be unable to repair the constructed deletion mutations. A mutation in *rec*A will also make the strain sensitive to ultra-violet light and will be an important containment factor.

In addition, it would prevent the strain from repairing the genetic lesions by homologous recombination using wild-type DNA found in the environment or the tissues of the host (commensal *Neisseria* for example). A *rec*A mutation also increases the general stability of the strain by reducing the loss of phenotypic characteristics by deletions and inversions which are mediated at least in part by such recombination pathways. *Rec*A genes have been widely studied in the art and their sequences are available. Reference may be made to EMBL/GenBank/DDBJ databases. 1992. ID code NM446REC (or Molecular Microbiology (1992) 6:2135-2146).

In many cloning procedures, selectable markers are used to select desired mutants. However, it may be preferred to remove subsequently the selectable marker. Since introducing a *recA* mutation will disable the recombination apparatus of the bacterium it will not be possible to use a subsequent *recA*-dependent recombination event to remove the selectable marker. Thus a particularly preferred method for introducing a *recA* mutation which allows subsequent removal of a selectable marker is to flank the selectable marker sequences with sequences which are recognised by a recombinase/resolve-type enzyme. For example, the product of the *parA*

gene is a resolvase that specifically excises regions of chromosomal DNA flanked by 140 bp *res* sites (J. Bact. (1995) 177:52-58). Therefore the *recA* mutation cassette may comprise a selectable marker gene flanked by *res* sites. Once mutant bacteria with the desired *recA* mutation have been selected, introduction of a plasmid encoding the *parA* resolvase will result in excision of the selectable marker gene. Plasmid RP4 (J. Bact. (1995) 177:52-58) is an example of such a plasmid. Expression of the resolvase protein even at low efficiency will be sufficient to result in the excision of the selectable marker gene flanked by the *res* sites. The plasmid bearing the parA gene lacks a Neisserial origin of replication and would be lost naturally from *Neisseria* sp.

Additional genetic stability.

Once the recA activity has been abolished, the bacterial strain may still have some residual ability to take up exogenous DNA. This may vary from strain to strain. If the frequency of such events is considered too high (e.g. by regulatory authorities) a further mutation may be introduced to reduce DNA uptake. Such a mutation may be made in any gene involved in DNA uptake and would be made in a particular strain prior to the mutation of *rec*A if it is found that the *rec*A mutation alone is not sufficient.

The *com*A gene of *N. gonorrhoeae* is involved in competence. A mutation in the *com*A locus would render this organism unable to take up exogenously added DNA, making the strain genetically isolated, biosafe and more stable. Corresponding mutations at homologous loci may be made in *N. meningitidis* and other organisms. Those of skill in the art will appreciate that it will be necessary to recombine the *rec*A and *com*A (or other gene involved in DNA uptake) constructs simultaneously, since both recombination and competence functions to introduce these lesions. Thus where a *rec*A and/or *com*A lesion is required in a strain, this/these will be introduced as the final genetic modification, following the other modifications described herein.

RTX toxin.

The *N. meningitidis* vaccine strains produced in accordance with the embodiments of the invention described above will still express the genes for the RTX toxin and its secretion machinery (encoded by *frp* locus). The outer membrane proteins for toxin secretion may be important protective antigens. However, the toxin itself, which is iron regulated, may cause some adverse reactions, for example in some subgroups of patients or with some attenuated

strains. If the production of the RTX toxin causes problems during the limited colonisation of the vaccine strain, then the toxin gene will be inactivated by defined deletion or other modification. This will leave the genes for the outer membrane transport proteins intact, since they may be important elements for the efficacy of the vaccine.

One option would be to inactivate the toxin structural gene (*frp*A) by the insertion of a *Sac*RB cartridge from *B. subtilis* . This would result in a sucrose sensitive phenotype which would provide a molecular tag, allowing genetic stability to be monitored during manufacture. The RTX toxin gene may be found by reference to Mol. Micro (1993) **9**:85-96.

Although an RTX mutation is envisaged primarily with reference to *N. meningitidis*, many other bacteria have a similar RTX toxin and corresponding mutations may be made in these. In addition, other bacteria produce other types of toxin and it may be necessary to introduce mutations into the genes encoding these toxins, using standard techniques.

Membrane Vesicles.

In addition to the live attenuated vaccine, membrane vesicles derived from this *N. meningitidis* or other vesicle-producing bacteria may be a better option for certain patients or patient groups. *N. meningitidis* and other bacteria naturally produce membrane vesicles which may be isolated. These may be used directly to induce a strong immune response.

Mutations in the *E. coli min*B locus (see Eur. J. Biochem. (1981) **116**:331-335) cause aberrant cell division and the production of mini DNA-less cells. Mutations in this locus and homologous loci in other bacteria will greatly increase the production of membrane vesicles or mini cells by these bacteria. Thus the present invention also provides a bacterium with additional mutations in loci homologous to the *E. coli min*B locus (described in Cell (1989) **56**: 641-649 – Genbank Accession No. J03153).

Strains of *N. meningitidis.*

To provide attenuated strains of *N. meningitidis* any suitable starting strain may be used. In general, clinical isolates are preferred since these represent strains which have been shown to cause disease in patients. Clinical isolates include KH 454 Serotype B15 P1.7 (J. Med. Microbiol. (1995) **42**:353-361), nm3474 Serotype B2B P1.10 (available from Nottingham Public Health Laboratory Service, Nottingham, UK) and strain B16:B6 (Schryvers *et al*, Microb. Pathog. 24, 75-87, 1998). However these are only examples and many clinical isolates

are available in medical microbiology laboratories throughout the world.

Whatever starting strain is used, preferred genotypes of attenuated strains in accordance with the invention include the following:

1. *aro*B, *gal*E, *lac:fur* fusion, *rec*A.
2. *aro*L, *gal*E, *lac:fur* fusion, *rec*A.

In either case one or more of the following characteristics are additionally preferred:

i) a mutation in a homologue of *min*B

ii) an RTX negative phenotype; and

iii) an *opc* gene, the expression of which has been modified or eliminated.

iv) a mutation in a capsule gene.

N. meningitidis as a carrier for other antigens.

Attenuated strains of the invention will also be useful as a vehicle for the delivery of other antigens from other pathogens, including viral and bacterial pathogens. This will be useful where protection against such other pathogens can be obtained by stimulation of the immune system by such an antigen. Examples of this include the hepatitis B surface antigen, pertussis toxin, *V. cholerae* or ETEC B subunit toxin or diphtheria toxin.

In these cases a gene encoding the antigen will be operably linked to a promoter capable of functioning in the bacterium of the invention to provide an expression cassette or vector and such a cassette or vector will be introduced into the bacterium. The cassette or vector may also comprise genomic sequences from said bacterium to allow for homologous recombination of the cassette or vector into the genome of the cell. For example, the cassette or vector may be designed such that the antigen gene replaces one of the genes described above which is to be attenuated.

Growth of strains and production of vaccines.

Once bacteria have been engineered in accordance with the invention, they may be grown in culture and the culture recovered to provide bacteria for a vaccine composition. Conditions for the growth of bacteria are well known in the art, and industrial processes for recovery of bacteria are also widely available.

Vaccine preparations.

The bacteria of the invention, or the membrane vesicles, may be formulated into vaccine preparations. Typically vaccines are prepared by mixing the bacteria with a pharmaceutically acceptable carrier or diluent. Such carriers or diluents include water, saline, dextrose, trehalose, glycerol, ethanol or the like, or mixtures thereof. The vaccines may comprise one bacterial strain or a mixture of strains (i.e. either a mixture of different types of the same species and/or different species).

The concentration of the attenuated strain in the vaccine will be formulated to allow convenient unit dosage forms to be prepared. Concentrations of from about 10^4 to 10^9 bacteria per ml will generally be suitable, e.g. from about 10^5 to 10^8 such as about 10^6 per ml. Where membrane vesicles are formulated, suitable concentrations will be in the range of from about 1 to 100, e.g. 5 to 50 mg of protein per ml.

The vaccine composition may, for example, be formulated as an aerosol, particularly for *N. meningitidis*, at concentrations the same as those described above and as a preparation for injection.

Administration of vaccines.

The vaccines of the invention may be administered to recipients in order to protect them against diseases caused by the corresponding wild type organism. Although human recipients are primarily envisaged - especially for *N. meningitidis* which causes disease in humans - vaccines of the invention will have veterinary uses, for example in vaccinating domestic pets such as cats and dogs, and in vaccinating livestock such as mammals including cattle, sheep and swine or other vertebrates such as poultry. These animals may be vaccinated in particular with attenuated enteric bacteria of the invention.

The vaccine may be administered by any suitable route. In general, subcutaneous or intramuscular injection is most convenient. This will lead predominately to a serum immune response which should prevent dissemination of the organism, throughout the body and the resulting septicaemia

Because it is unknown whether this route of administration will provide any protection against colonisation of the nasopharynx and carriage, vaccinations against bacteria which also invade through this route may also be by use of an intranasal spray. This will lead to

colonisation of the nasopharynx mimicking closely the natural route of infection and carriage. The expected immune responses will be (a) mucosal - which would generate mucosal antibodies and should prevent colonisation by pathogenic bacteria and (b) serum - which should prevent dissemination of the bacteria throughout the body and the resulting septicaemia. This route of administration is particularly preferred for vaccination with attenuated strains of *N. meningitidis.*

Indeed, for *N. meningitidis* it is intended that this vaccine strain will primarily be delivered as a nasal aerosol, allowing the strain to colonise the nasopharynx (the initial site of colonisation for the wild-type organism). Due to the *aro* and the *gal*E mutations the strain will exhibit a limited invasion of the tissues and possibly entry into the blood stream before viability is lost through serum killing and autolysis. This will mimic closely the progression of the natural route of infection, producing mucosal and serum immunity. This may be boosted if desired by injection.

For infants where there may be some reluctance to vaccinate with a live attenuated vaccine, non-viable membrane vesicles may be used. As described above, such vesicles are naturally produced by *N. meningitidis* and can be harvested as a vaccine to be administered subcutaneously, intramuscularly or by nasal aerosol.

Doses of vaccine will ultimately be at the discretion of the physician, taking into consideration the status (e.g. age, weight, disease status) of the recipient and the nature of the vaccine strain used. However some general guidance may be found by reference to the published literature.

For example, intranasal doses of *N. meningitidis* may be calculated by reference to animal models such as those disclosed in Microbial Pathogenesis (1992) **12**:415-420 or Canadian Journal of Microbiology (1984) **30**:1022-1029. Suitable intranasal doses are around 10^5 to 10^8, e.g. about 10^6 or 10^7 organisms in a single dose.

Live attenuated organisms may be administered subcutaneously or intramuscularly at up to 10^8 organisms in one or more doses, e.g. from around 10^5 to 10^8, e.g. about 10^6 or 10^7 organisms in a single dose.

Membrane vesicles may also be administered subcutaneously, intramuscularly or nasally in an amount of about 25-100 mg of protein (depending on LPS content of vesicles) in 2-3 doses. (See for example Infection and Immunity (1996) **64**:2745-2751, Infection and Immunity (1995) **63**:4642-4652 or Infection and Immunity (1995) **63**:3531-3536).

The recipient may receive one or more doses, in the latter case the doses may be repeated at an interval of from one week to one or more months, e.g. 2, 3, 6, 12, 18 or 24 months.

The above doses are provided primarily with reference to adult humans. They may be scaled up or down in proportion to body weight for non-adults or for other species.

The invention will be described with reference to the following Examples, which are intended to be illustrative only and non-limiting.

EXAMPLES

Materials and Methods

Gene construction

The following PCR primers were synthesised for use in the genetic construction of vaccine strains. The primers were designed to contain a restriction site for ease of manipulation of the amplified products.

PRIMERS

Name - Gene - Sequence - Direction - Enzyme

T G

aro1 - *aroA* - GGAATTCGATTCCGATGATATCCGTCATATG - forward - *EcoRI*

CAG C CG G C C

C

Aro2 - *aroA* - GGAATTCATCAGGAATATGATTCATATCCAT - reverse - *EcoRI*

G G G G G G

AroB1 - *aroB* - GCAGATGCCCGAAGCTTTTTATAGCGG

AroB2 - *aroB* - GAGCTCGGTACCGTGCAGCGTGTCCAGATCTGCAAG

AroB3 - *aroB* - CATAAAGGGATCCTGGTTCGCCAG

AroB4 - *aroB* - GGTACCGAGCTCCAAATGAAGGCAGATCTCGTCGCCC

AsdA - *asd* - ACAATGAAAGTAGAATTCGTCGGCTGG - forward - *EcoRI*

C

AsdB - *asd* - GAATGCGGAGATGAATTCGCCGCCCAT - reverse - *EcoRI*

G C T

FurD - *fur* - CCCTGCTCACGTCGACCAG - reverse - *Sal*I

FurE - *fur* - ACGCGGTCGACGCTGCACG - forward - *Sal*I

FurF -*fur* -AATACGCAATTGGATCCTGCTTGC - reverse - *Bam*HI

FurG - *fur* - GATATTGAATCATATGGAAAAATTC - forward - *Nde*I

galEA - *galE1* - GTGATTTTGGATAAGCTTTGCAATTCC - forward - *Hin*dIII

galEB - *galE1* - CCAGCGCCATGAAGCTTCCATCAT - reverse - *Hin*dIII

lac1 - *lacI* - GACAGGATCCAATGGTGCAAAACC - forward - *Bam*HI

lac3 - *lacZ* - AATCATGGTCATATGTGTTTCCTG - reverse - *Nde*I

recA1 - *recA* - CGGAATTCGGTCTGAAGCGGATG - forward - *Eco*RI

recA2 - *recA* - CGCAGCAGGAATTCCCGTTTATCG - reverse - *Eco*RI

These primers were used for amplifying target DNA to obtain copies of wild-type genes

from target starting strains of bacteria. To obtain target DNA, 80 ml of overnight culture were grown in broth and harvested in 2 x 50 ml polypropylene tubes. The bacteria were recovered and DNA obtained using a standard phenol-chloroform extraction protocol.

Target genes were amplified by PCR under appropriate conditions carried out for 35 cycles, with the final cycle having an extension at 72°C for 9.9 minutes so that all extensions are completed. The purity, size and amount or PCR product obtained from any amplification is determined simply by agarose gel electrophoresis. 10 µl of the PCR reaction are analysed by agarose gel electrophoresis on a 1% agarose gel and different size DNA fragments separated and identified under UV light by staining with ethidium bromide. The size of the amplified DNA fragment was determined by comparison with known size markers. The amplified product was cloned into one of a range of vectors including pUC19, pCRII, PCRscript and pSP72.

The above techniques may all be found in standard reference books such as Sambrook *et al..*, Cold Spring Harbor, 1989.

Mutants were constructed *in vitro* by identifying restriction enzyme sites from the information obtained by the sequencing of the cloned PCR product. Once restriction site(s) in the desired area(s) are identified, the cloned fragment is cut with the appropriate enzyme(s), removing a fragment from the centre of the cloned insert. The plasmid is then re-ligated and deletion derivatives identified by the reduction in size on agarose gels and by sequencing. This procedure is used to develop defined mutations in the genes *aro*A, *gal*E, *asd* and *rec*A.

Vectors

Vectors used for transformation of *N. meningitidis* include uptake sequences to assist with uptake of the vector by the bacterium. These sequences comprise a 10-mer inverted repeat (Goodman and Socca, Proc. Natl. Acad. Sci. (1988) 85:6982-6986). The nucleotide sequence of the uptake sequence used is shown below, with the inverted repeats underlined in bold.

GGGCCCGGGCTGCA**GCCGTCTGAA**ATGCAT**TTCAGACGGC**TGCAGCCCGGGCCC

Transformation of *N. meningitidis.*

N. meningitidis grown overnight (37°C, 5% CO_2) on GC media (Difco) supplemented with 1% Vitox (Oxoid), are gently resuspended in protease peptone broth (15 g/l protease

peptone No.3 (Difco), 4 g/l K_2HPO_4, 1 g/l KH_2PO_4, 5 g/l NaCl, 1% vitox) containing 10 mM $MgCl_2$ and diluted to an OD_{600} of 0.2. ~~0.5 g~~ 0.5 µg of plasmid is added to 0.5 ml of resuspended bacteria and incubated (37°C, 5% CO_2) for 4 hours. ~~100 l~~ 100 µl aliquots are plated out on selective plates (GC agar containing antibiotics, and any other nutritional supplements required; see table below). Plasmids used for transformation are suicide vectors with various selectable markers (see table) and a synthetic uptake sequence specifically required for natural transformation of *Neisseria* sp.

Table: Media supplements for selection of mutants

Supplement	**Working conc.**	**Use**
Chloramphenicol	12 g/ml	Selection of *cam* containing mutants
Erythromycin	5 g/ml	Selection of *ery* containing mutants
Kanamycin	MIC*	Selection of *kan* containing mutants
Streptomycin	10 mg/ml	Selection of streptomycin sensitive mutants
Sucrose	15%	Selection of *SacRB* deficient mutants
Fe nitrate/sulphate	25 M	Optional for *fur/lac* mutants
IPTG	0.1mM	Optional for *fur/lac* mutants
Diaminopimelic acid	100 g/ml	Supplement for *asd* mutants

*Minimum inhibitory concentration

The final vaccine strain should preferably not contain any genes encoding antibiotic resistance. These genes are used as a marker of recombination events and may be removed by subsequent recombination.

Following introduction of the mutants into host bacteria the introduction may be confirmed by Southern blotting techniques under standard conditions, using the PCR amplified gene as a probe.

Example 1: *aroB* mutant (Figures 1 to 3)

1. Preliminary work

It was hypothesised that the *N.meningitidis aroB* gene would be almost identical to the *N.gonorrhoea aroB* gene. Hence, the *N.gonorrhoeae* genome database was searched

using the protein sequence from the *E. coli aroB* gene using tblastn. This located an open reading frame on Contig 306 (which recently evolved into Contig 310) encoding a very similar protein, a putative gonococcal *aroB* gene.

Based on the gonococcal Contig 306 sequence, primers AroB1 and AroB2 were designed to PCR approximately 1kb DNA upstream plus some of the beginning of the putative *N.meningitidis aroB* gene. Primers AroB3 and AroB4 were also designed to PCR some of the end of the *aroB* gene and approximately 1kb of downstream sequence. Restriction sites were added so that the two PCR products could be cloned and joined to create a version of the *aroB* gene with the central portion of the gene missing. It was planned to replace the chromosomal meningococcal *aroB* gene with this deleted version using homologous recombination and so make an *aroB* mutant.

2. Cloning and sequencing of the putative meningococcal *aroB* gene

Genomic DNA from a streptomycin resistant version of the *N.meningitidis* clone B16:B6 (Schryvers *et al*, Microb. Pathog. 24, 75-87, 1998) was used for cloning the *aroB* gene. PCR products AroB1-2 and AroB3-4 were generated using Taq and cloned into the pCRII vector (Invitrogen) for storage and sequencing. Resulting plasmids were labelled pCR1-2 and pCR3-4 respectively. Preliminary sequencing results (sequencing ends of PCR products) confirmed they contained the target sequences.

3. Construction of suicide vectors for creating an *aroB* meningococcal mutant

The suicide vectors are based on vector pGIT5. This was derived from the pCRII cloning vector (Invitrogen) but contains a deletion in the ß-lactamase gene for safety considerations and also a synthetic gonococcal uptake sequence cloned into the EcoRI site to guarantee uptake of the vector into *Neisseriae* species. In more detail, the ampicillin gene of pCRII was knocked out. A consensus DNA uptake sequence was synthesised based on *N.gonorrhoea* data (Burnstein *et al*, J. Gen. Microbiol. 134 (Pt. 3) 547-557, 1988: Stein, Can. J. Microbiol. 37, 345-349, 1991). The uptake sequence was cloned into the EcoRI site of the pCRII.

The *aroB* deletion strategy involved two steps and used an erythromycin resistance (erythromycin R) cassette. Using a two-step process, the *aroB* gene could be replaced by the deleted version without leaving an antibiotic marker in the gene. The strategy did require a

streptomycin-resistant *N.meningitidis* strain, however.

The first suicide vector contained the erythromycin R cassette sandwiched between the AroB1-2 and AroB3-4 PCR products. Firstly, AroB3-4 was ligated between the KpnI and BamHI sites of pGIT5 to produce pGIT5aroB3-4. AroB1-2 was then ligated between the KpnI and HindIII sites in pGIT5aroB3-4 to make pGIT5aroB Δ1-4. Finally, the erythromycin R cassette was inserted into the blunted KpnI site of pGIT5aroB Δ1-4 to produce pGIT5aroB Δerm.

The second suicide vector is the same as the first, but omitting the erythromycin R cassette. This had already been made and was pGIT5aroB Δ1-4.

4. Interruption of the N.meningitidis aroB gene using homologous recombination

The streptomycin-resistant version of the *N.meningitidis* B16:B6 strain (B16B6str^R) was transformed separately with two clones of the pGIT5aroB Δerm vector and four erythromycin resistant clones picked and stored from each transformation (ie. probably eight identical clones). All eight tranformants were erythromycin resistant and streptomycin sensitive. Hence, the pGIT5aroB Δerm cassette has almost certainly integrated into the bacterial chromosome. Kanamycin susceptibility was not tested, but would confirm the absence of the pGIT5 plasmid. The transformants have been cultivated on Blood Agar and GC agar plates on the assumption that both contain enough aromatic amino acids to overcome the attenuation of the predicted *aro* mutation. Minimum essential medium MEM with agar (with and without Vitox) plus all the amino acids has been tested as a minimal medium, but none of the transformants grew and control strains did not grow well.

Example 2: *asd* mutant (Figure 4)

A fragment comprising approximately 90% (1.3 kb) of the *asd* gene was amplified using PCR. Primers asd1 and asd2 were designed by back-translation of the protein sequence based on the codon usage of *N. meningitidis*. *Eco*RI sites were included at the 5' ends of the primers to facilitate cloning into the plasmid pUC19. The resulting plasmid pASD-1 was sequenced to confirm the identity of the cloned fragment and to generate a restriction map.

Restriction sites were identified to create a deletion in the middle of the gene which also throws the latter part of the gene out of frame. The insert removed was a *Bcg*I fragment from positions 471-586 of the gene (where 1 is the A of the ATG start codon). This ensured complete

inactivation of the gene product. A selectable marker cassette (comprising either CAT and *Sac*RB genes which confer chloramphenicol resistance and sucrose sensitivity, or Erm and Rps1 genes which confer erythromycin resistance and streptomycin sensitivity (Johnston, 1996, pages 37-38 in Abstracts of the 10th International Pathogenic Neisseria Conference: Sept. 8-13 1996, Baltimore, MD, USA; eds. Zollinger *et al.*)) was inserted at an *Nsp*I site of the undeleted *asd* gene (this occurs within the *Bcg*I deletion site). This construct was then used to transform *N. meningitidis*, with positive selection of mutants (resistance to chloramphenicol or erythromycin). Resistant colonies were then purified and transformed for a second time using the deletion construct devoid of any markers, with positive selection using either resistance to sucrose or streptomycin.

Mutants which have a mutation in the *asd* gene which encodes the essential cell wall enzyme -semialdehyde dehydrogenase can be identified by their requirement for diaminopimelic acid (DAP). Putative *asd* mutants were replica plated onto DAP-deficient and DAP-replete media. Those strains which grew on DAP-replete medium (100 g/ml) but not on DAP-deficient medium were *asd* mutants.

Example 3: *fur* construct (Figure 5)

For alterations in the *fur* gene it was essential not to have a complete knockout mutant since this may be lethal. Therefore the *fur* gene was placed under the control of another promoter which could be switched on or off independently of the factors (iron) which normally controls *fur* expression.

Primers for the amplification of the entire *fur* gene were designed from the published sequence with an *Nde*I restriction site in the forward primer (primer furG) at the transcription start of the gene and a *Sal*I restriction site in the reverse primer (primer furD) at the distal end of the *fur* gene. Primers furD and furG were used for the amplification of a 600 bp fragment containing the entire *fur* coding sequence using PCR. The DNA fragment was polished with *Pfu* polymerase and cloned into the vector pCR-script (Stratagene), to give the plasmid pFUR-DG.

Primers for the amplification of a 300 bp region upstream of the *fur* gene were designed with a *Bam*HI restriction site in the reverse primer (primer furF), proximal to the transcription start and a *Sal*I restriction site in the forward primer (primer furE), distal to the transcription start. The 300 bp DNA fragment was cloned into the vector pCR-script as above to give the plasmid pFUR-EF.

Finally, two primers were designed and constructed to the *lacZ* promoter and repressor region (*lac*I) of the *E. coli lac* operon. The forward primer, lac1, contained a *Bam*HI site and the reverse primer, lac3, included the *Iq* mutation and an *Nde*I site which would regenerate the ATG transcription start when ligated to the *Nde*I site of the gene pFUR-DG plasmid insert. The fragment generated using these primers was cloned into the vector pCRII (Invitrogen), to give pLAC-13.

The Fur-EF fragment was removed from pFUR-EF by cutting with *SalI* and *BamH1*. The Lac13 fragment was removed from its vector with *Bam*HI and *Nde*I. The Fur-DG fragment was removed from pFUR-DG by cutting with *Nde*I and *Sal*I. These were sequentially ligated together to give the complete *fur/lac* construct in pUC19. This was then sub-cloned into the pGIT5 suicide vector and inserted into the chromosomal location of the wild-type *fur* gene by allelic exchange.

In more detail, the *fur/lac* construct was cut from the construction plasmid pUC19-*fur/lac* using the enzyme *SalI*. This was made blunt ended by filling the in 5' overhangs using klenow. The now blunted *fur/lac* fusion was cloned into the blunt *EcoRV* site of pGIT5 to give the plasmid pGIT5*fur/lac*. PGIT5*fur/lac* was transformed into the *E.coli* host DH5α. A 100 ml culture of DH5α (pGIT5*fur/lac*) in LB broth was grown up at 37°C to an $OD_{600} = 0.7$. This was used to carry out a large-scale plasmid preparation by the alkaline lysis method.

PGIT5*fur/lac* was introduced into the *N.meningitidis* strain B16:B6 as follows. *N.meningitidis* strain B16:B6 was grown in proteose-peptone broth at 37°C to an $OD_{600} = 0.2$. These were pelleted by centrifugation and resuspended in an equal volume of proteose-peptone broth containing 10 mM $MgCl_2$. To 250 μl of bacterial suspension in a Bijou a maximum the pGIT5*fur/lac* was added to a final concentration of 1 μg/ml. This was incubated at 37°C for 4 hours in an atmosphere of 5% CO_2 in air with the lid of the culture vessel loose to allow CO_2 entry. After this time the culture was plated onto GC agar plates and allowed to grow at 37°C in 5% CO_2 in air until colonies showed.

Colonies were replicated/transferred to nylon filters and colonies lysed by floating the membrane on a solution of 1% SDS. The plates were re-incubated to allow the colonies to grow up again. The DNA from the colonies on the membrane was denatured by floating on a 1N solution of NaOH and then neutralised by floating on 1M Tris pH7.0. The filters were UV irradiated to bind DNA to the membrane and then hybridised to a dig-labelled *lacIR* fragment prepared by a commercial kit (Promega). After hybridisation and extensive washing the

membranes were developed using the solutions and protocol provided and *N.meningitidis* strains containing a *lacIR* fragment showed up as dark spots on the filter. The corresponding colonies were picked from the re-grown agar plates, grown up and stored. Confirmation of the correct construct was carried out by PCR amplification of sequences from chromosomal DNA using the *fur* and *lac* primers to confirm the presence of the fusion and its location. This will be confirmed by the Southern blots of restriction digests of chromosomal DNA.

One of the *fur/lac* strains were picked and grown for 12 hours in proteose peptone broth with varying concentrations of IPTG. Total proteins were prepared from the cultures by boiling in SDS-PAGE loading buffer (without the methylene blue dye). Lysates were dotted onto nitro-cellulose membranes and fixed in 1-% trichloro-acetic acid solution. The membranes were incubated for 1 hour with a 1:1000 dilution of a rabbit polyclonal antibody to tranferrin binding proteins Tbp1 and 2, in 50 mM Tris pH7.0

The membranes were washed extensively in 50 mM Tris pH7.0 and then incubated with a goat anti-rabbit secondary antibody, conjugated to alkaline phosphatase (Sigma), in 50 mM Tris pH8.0 for 1 hour. After this time the membranes were washed in 50 mM Tris pH8.0 and developed in BCIP/NBT solution (available as tablets from Sigma). Binding of primary antibodies was seen as an intense blue/black staining in the region of the applied proteins, no antibody binding was seen as little or no colour development.

The results showed that the primary antibody to Tbp1 and 2 bound more strongly to cells in the presence of IPTG than in the absence. Without IPTG there was very little binding of antibody (comparable to wild type in high iron conditions) indicating little expression of the *fur*/iron regulated Tbp 1 and 2. Whereas the concentration of IPTG was increased the binding of the primary antibody increased up to a level which appeared comparable to that seen with the wild type in low iron or iron free conditions. These results show that the *fur/lac* fusion strain does express its iron regulated proteins in response to IPTG. These data can be confirmed by Western blot analysis of total proteins and outer membrane proteins using various antibodies to iron regulated proteins. This could also be done for whole cells by colony blots and immunogold electron microscopy to confirm that the proteins are localised properly. Also, comparison of outer membrane profiles of iron-induced wild type IPTG induced fusion strains will show whether the profiles are similar.

Example 4: *galE*1 mutant

N.meningitidis contains two chromosomal *galE* gene sequences, *galE1* and *galE2* at separate loci. *GalE1* is complete, expressing a functional gene product, while *galE2* is truncated and lacks homology over the first third of the protein. Primers galEA and galEB were designed to areas which are not conserved between *galE1* and *galE2* to amplify a 1.5 kb fragment of *galE1*. A *Hind*II site was engineered into each primer to facilitate cloning into pUC19. After subcloning into the suicide vector pGITB (a pUC based vector with neisserial uptake sequence and lacking a *Bpm*I site in the inactivated ampicillin gene, a selectable marker cassette (see Example 1 for alternatives) was inserted at a *Bpm*I site (very close to the *Bcg*I deletion site). This construct was then used to transform *N.meningitidis*, and mutants identified by a chloramphenicol or erythromycin resistant phenotype. Resistant colonies were then purified and transformed for a second time using the *GalE1 Bcg*I deletion construct devoid of any markers, with positive selection using either resistance to sucrose or streptomycin.

To establish a change in LPS type, LPS is prepared from mutants and parental wild-type *N.meningitidis* by a proteinase K digestion of OMPs method and analysed on 15% tricine SDS-PAGE gels. Bands are visualised by silver staining. LPS from *galE* mutants demonstrate greater electorphoretic mobility than their wild type parent strains.

Example 5: *rec*A mutant.

DNA sequences of *rec*A genes from various bacterial species were compared and primers recA1 and recA2 were designed within conserved regions of the gene using the codon frequency of *N. meningitidis*. The primers were designed with *Eco*RI restriction enzyme cleavage sites to facilitate cloning. The primers were used to amplify *rec*A from *N. meningitidis* and the product was cloned into pUC19. The resulting plasmid, pRECA-1, was partially sequenced to verify that the insert was from the *rec*A gene and restriction mapped to identify useful restriction enzyme sites for the generation of the deletion derivative. This deletion derivative, pRECA-D, was recombined into the wild type *N. meningitidis* chromosome and the resulting strain was tested to determine its sensitivity to UV light compared to the parental strain.

UV sensitivity compared to the isogenic wild type strain can be determined by inoculating blood agar plates with parallel streaks of the wild type and the putative *rec*A mutant. The inoculated plates are exposed to known UV energy for increasing times. The plates are

incubated at 37°C and 5% CO_2 in air for 24 hours. UV sensitivity is shown by reduced growth of the putative mutant on plates compared to the adjacent wild-type strain.

The strains obtained by this procedure are effectively isolated genetically and no further construction can be undertaken since they are now deficient in homologous recombination.

Example 6: Δ*aroB, lac:fur* fusion mutant

The procedure of Example 3 is repeated, but this time using the attenuated *N. meningitidis aroB* mutant obtained by the procedure of Example 1. This provides an attenuated Δ*aroB*, *lac/fur* fusion mutant of *N.meningitidis.*